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Conditioned placebo effect in dogs decreases separation related behaviours

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ABSTRACT

In humans, placebo effect can be produced by giving verbal information and also by conditioning when, after repeated administration of an active substance, an inactive compound that just looks like the drug administered before, can produce the effect of the active substance. Conditioned placebo effect has been reported in rodents, however, the dog (*Canis familiaris*) may also provide a promising model species. In our study dogs' behaviour was observed while they were repeatedly separated from their owners in the same unfamiliar room. First, subjects did not receive any pre-treatment (Baseline trial), then they participated in either of two different conditioning contexts: after having received either sedative drug (Conditioned group) or non-sedating vitamin (Control group) treatment, subjects participated in 3 conditioning trials on consecutive days. Finally, in the 'Test trial', both groups were separated from their owners after receiving placebo (non-sedating vitamin). Results show significant effect of the sedative drug conditioning; when comparing the change from Baseline to Test trials in the *Conditioned* and the *Control* groups, conditioned subjects showed less active signs of distress ($U_{(26)} = 48$, $p = 0.021$) and more passive behaviours ($U_{(26)} = 50$, $p = 0.027$). We also investigated the association between dogs' susceptibility to conditioned placebo effect and their expectancy bias towards positive outcomes and found a positive correlation ($r_{(12)} = 0.697$, $p = 0.008$), suggesting that dogs with more positive expectations are more responsive to placebo treatment. Considering previous human findings about stronger responsiveness to placebo in optimistic people, our results support the validity of the application of a dog model towards a better understanding of some aspects of the placebo phenomena in humans.

1. Introduction

Investigation of the mechanisms as well as the behavioural and psychological dimensions of the placebo effect has become a burgeoning field of life sciences in the last few decades. According to the widely accepted definition, placebo is a substance or procedure that has no inherent power to produce an effect that is sought or expected (Stewart-Williams and Podd, 2004). The effect that placebos have can be highly variable involving both

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psychological and physiological changes (e.g. endogenous opiate release Petrovic et al., 2002; Wager et al., 2004).

Nevertheless, placebo effect is often conceptualised as a psychosocial context effect (Benedetti and Colloca, 2004) involving the formation of cognitive expectancies, a process driven by verbal information from a trustworthy, certified person (Benedetti et al., 1999, 2003). Although this view would strongly suggest that placebo effects are limited to humans, experimental evidence indicates that this complex phenomenon stems from both higher mental functions and lower conditioning effects, and thus can also be studied in nonhuman subjects (see Price et al., 2008 for a review).

Increasing evidence suggests that placebo responses can be formed by classical conditioning in both humans (Voudouris et al., 1990) and different species of animals (McMillan, 1999). This process is based on the association between an active substance (unconditioned stimulus) and some characteristic property of the substance (smell, taste, colour) and/or some environmental cues (places, persons, procedures, rituals) surrounding the treatment (conditioned stimuli). After repeated experience of the specific effects of the treatment, a procedure with the same features but without the active substance can produce the very same physiological and/or behavioural effects evoking a conditioned response. The induction of a placebo effect via conditioning is possible even when the effect of the treatment is unconscious and imperceptible to the subject (e.g. change in hormone level – Benedetti et al., 2003 or immune response – Goebel et al., 2002).

In addition to rats and other laboratory rodents that are often used to demonstrate the conditioned placebo effect (see Stewart-Williams and Podd, 2004 for a review), some evidence suggests a placebo-like effect in pet dogs that have undergone veterinary treatment. However, it is important to note that in all placebo studies on dogs, assessment of the magnitude of placebo responses has been based solely on the owners' subjective evaluation; therefore, the results could be strongly influenced by the owners' expectations (Muñana et al., 2010; Jaeger et al., 2005). Although the mechanism mediating the effects of placebo treatment in dogs is still unclear, Cracknell and Mills (2008) investigated the role placebo treatment plays in overcoming fear and anxiety. They found a significant anxiolytic effect in dogs that showed excessive fear response to fireworks. This result was also based on owners' reports, so further confirmation of conclusions about the role of placebo in alleviating fear or relieving pain would require the collection of behavioural data through direct observations.

These findings are in line with the increasing evidence of dogs' human-tuned social cognitive skills (Kaminski, 2008) and support the idea that the fear/anxiety-alleviating effect of placebo treatment in dogs is a phenomenon worth investigating within the context of the dog–human social bond. It has been suggested that dogs possess a specific behaviour organising mechanism (called interspecific attachment), which evokes specific responses in stress situations related to separation from the attachment figure (see Topál and Gácsi, 2012 for a review). Separation related behaviours, the fear or dislike of isolation from the owner even in familiar environments, are frequently reported problems in pet dogs (Wright and Nesselrote, 1987). Behaviour symptoms associated with physiological changes (Palestrini et al., 2010) can be reduced by medication or behaviour therapy (Butler et al., 2011; Appleby and Pluijmakers, 2004). Concerning the medication to treat anxiety disorders in dogs, Sedalin is one of the widely used psychoactive drugs. Its active substance is acetylpromazine, which has a tranquilising effect (Booth and Mc Donald, 1991) as it causes a general depression of the nervous system characterised by both neuronal and behavioural changes (Tontodonati et al., 2007).

The most widely used experimental paradigm to study dog–human attachment and separation anxiety is the Strange Situation Test (SST), which capitalises on the tendency of dogs to show specific behaviours when separated from the owner in an unfamiliar room (Topál et al., 1998). In this context, efforts to re-establish the proximity (scratching the door, orientation to the door, vocalisation) are typical characteristics of dogs' behaviour (e.g. Prato-Previde et al., 2003; Palmer and Custance, 2008).

Although behavioural manifestations of separation anxiety in dogs are easy to observe and behavioural symptoms of anxiety can be reduced by tranquilisers, placebo conditioning studies are missing. Thus, in the first experiment of the present study we aimed to investigate the role of placebo in reducing dogs' separation related distress behaviours and to determine whether it is possible to produce a conditioned placebo-effect after repeated experiences of the anxiolytic effects of psychoactive drug (Sedalin) treatment in the experimental situation.

Moreover, since responsiveness to expectancy based placebo treatment in humans is positively affected by subjects' dispositional optimism (Geers et al., 2005, 2007, 2010; Morton et al., 2009), in a follow up study (Experiment 2) we aimed to test whether individual differences in dogs' susceptibility to the placebo effect are linked to the subjects' tendency to form positive expectations about upcoming events.

Discrimination learning tasks are standardly used to assess positive expectation bias in non-human animals (Harding et al., 2004) including rats (Burman et al., 2009), sheep (Doyle et al., 2010), starlings (Bateson and Matheson, 2007), and honeybees (Bateson et al., 2011). After the subjects have learned that one stimulus (sound, colour, location, etc.) is negative (non-reinforced), while another one is positive (reinforced) they typically respond with higher latency to the negative stimulus. When subjects are presented with an ambivalent stimulus (transition between negative and positive stimuli), "optimistic" subjects respond as if they were presented with the positive stimulus (Mendl et al., 2009). This method was successfully applied for dogs with location cues (Mendl et al., 2010; Müller et al., 2012) and in colour discrimination contexts (Burman et al., 2011).

In the present study we hypothesised that there would be a significant positive correlation between dogs' susceptibility to placebo conditioning (measured by the relative change in behaviour signs of distress – Experiment 1), and their positive expectation bias scores (measured by Mendl et al.'s (2010) discrimination learning task – Experiment 2).

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2. Materials and methods

2.1. Experiment 1: conditioned placebo effect

2.1.1. Subjects

Participants were recruited on a voluntary basis. Owners completed a brief questionnaire about their dog's behaviour during different separation situations, and those dogs that were affected in at least 3 out of the 7 contexts, and were reported to show behavioural problems (e.g. excessive barking, salivating, destructive behaviour) when left alone in an unfamiliar place were selected. An additional criterion for selection was that the dog was not taking any medication and had no known health problem. All owners were provided with adequate information about the effects of Sedalin and they signed the informed consent form to participate. However, owners were not informed of the specific aims and design of the study, and they did not know if their dogs had been given Sedalin or vitamin before the trials. The procedure was approved by the Ethical Committee for Animal Experimentation of Eötvös University (No. XIV-I-001/521-4/2012), and conducted in accordance with the national laws regulating animal research.

Thirty-one adult (>1 year) pet dogs were included in the experiment, but 3 owners and their dogs did not come back to all trials. The remaining 28 dogs (mean age ± SD: 1.8 ± 3.09 years, 15 males and 13 females from 13 different breeds and 13 mongrels) were tested and included in the data analysis. Subjects were randomly assigned to either the *Conditioned* or the *Control* group ($N = 14–14$). The two groups did not differ in their mean age ($t_{(26)} = 0.905$, $p = 0.374$), sex ratio ($\chi^2_{(1)} = 0.144$, $p = 0.705$), breed distribution ($\chi^2_{(7)} = 3.0$, $p = 0.885$), body weight ($t_{(26)} = 0.786$, $p = 0.439$), separation anxiety questionnaire score ($U_{(26)} = 84$, $p = 0.541$) and in terms of duration from baseline to test trial ($t_{(26)} = 1.047$, $p = 0.305$), and duration from the last conditioning event to test trial ($t_{(26)} = 0.0$, $p > 0.999$).

2.1.2. Experimental arrangement

The experiment took place in a room (3.9 m × 4.1 m) at the Family Dog Project lab, at Eötvös University, Budapest. Only a chair and some toys for the dog were placed in the room. Two different doors were used by the two human participants, the owner and the stranger (Fig. 1). The stranger was always a woman who was unfamiliar to the dogs.

2.1.3. Procedure

Dogs participated in five trials, taking 1–4 day breaks (at least 24 h) between them.

2.1.3.1. Baseline trial. The procedure was identical for both groups. Subjects participated in a modified and shortened version of Strange Situation Test (Topál et al., 1998). It consisted of 3 episodes, each lasting for 2 min. Human participants (owner and stranger) followed detailed instructions that determined their behaviour during the test. The three episodes were preceded by a short introductory phase during which the experimenter introduced the dog and the owner to the experimental room through Door 2, and the dog was allowed to explore the room for 30 s. Then, the experimenter left the room with the owner through Door 2.

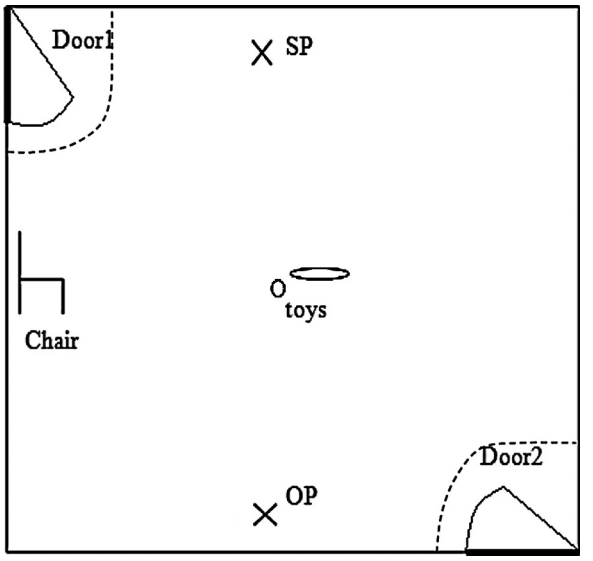

Fig. 1. Schematic layout of the experimental arrangement in Experiment 1. A chair and some toys were present in the experimental room. Door 1 was used by the stranger to enter; Door 2 was used by the owner to enter. The areas near the door are indicated with broken lines. SP & OP were places marked with adhesive tape on the floor where the stranger (SP) and the owner (OP) stood (see Section 2.1.3).

The episodes followed each other in a fixed order: the dog was (1) alone, (2) with a stranger, (3) with the owner in the experimental room.

Episode 1: Dog alone

The dog was left alone, and observed by the owner and experimenter on the monitor in the adjacent room (without speaking, thus the dog could not hear people in the adjacent room).

Episode 2: Dog and stranger

The stranger entered the room (through Door 1), stepped up to a predetermined point (SP) and stood there for 1 mine. She adjusted her behaviour to that of the dog (petted its head and back if the dog initiated contact) and tried to keep the dog away from the doorway by playing or petting (depending on the preference of the dog). After 1 min, she sat on the chair and stopped playing. During the second minute she was allowed to pet the dog if it initiated contact.

Episode 3: Dog and owner

The owner entered the room through Door 2 and stepped up to a predetermined point. Meanwhile, the stranger left through Door 1. The owner then greeted and comforted the dog (petting and playing – depending on the dog's reaction). The owner stood at the predetermined point (OP) until the end of the episode, playing with and/or petting the dog if it initiated.

2.1.3.2. Conditioning trials (2–4). In case of the three conditioning trials, 25 min before each trial, dogs received either a sedative drug (Sedalin Gel Oraldoser A.U.V. manufactured by Vetoquinol Biowet Sp.z.o.o., dose: 1 ml/35 kg body

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weight) in a piece of liverwurst (approx. 10 g, manufactured by Szegedi Paprika Zrt.) or a non-sedating vitamin formulation (dose: 1 ml/35 kg body weight, Canigest Paste manufactured by TRM Pet Products) in a piece of liverwurst. Sedalin is widely applied by veterinarians as tranquiliser and anaesthetic premedicant; it shows effects in 20 min and lasts 6–12 h. The vitamin did not have any effect during the experiments. Dogs received the treatment in the kitchen of the department and spent the 25 min there resting next to the owner.

In order to increase the saliency of 'treatment' and to facilitate the formation of associations between the physiological effects of pre-trial treatment and the unfamiliar test environment, we introduced an additional salient treatment right before the conditioning trials in both groups. The experimenter sprayed the dogs' muzzle and paws with clear water (using a hand pump spray bottle) and during the spraying she gave one more piece of liverwurst to the dog.

Conditioning trials included three episodes similar to the Baseline, however, the owner was present with the dog in all three episodes in order to avoid any possibility of separation from the owner being directly associated with the anxiolytic effects of Sedalin. Episodes 1 and 3 were identical to episode 3 in the Baseline trial. In episode 2, in contrast to the Baseline trial, the owner did not leave but was standing at the predetermined point and was allowed to interact with the dog while the stranger was in the room.

2.1.3.3. Test trial. In the test trial, all dogs were treated similarly. Both groups received placebo (vitamin treatment) in a piece of liverwurst 25 min before the trial. Their muzzles/paws were sprayed with water and they received one more piece of liverwurst right before the trial (Table 1). The procedure of this trial was identical to that described in the Baseline.

After the conditioning trials and test trial the owners' opinion about the type of treatment (Sedalin or placebo) their dogs received was asked.

2.1.4. Behaviour coding

As behaviours related to separation anxiety are typically displayed close to the exit/entry door (see e.g. Prato-Previde et al., 2003; Palmer and Custance, 2008; Palestrini et al., 2005), we recorded the durations of anxiety-related behaviours while staying close (<1 m) to the doors. The two doors were not differentiated because both could be considered as a potential exit by the dogs. On the other hand to examine the sedative effect of the drug the time spent passively was also measured. Relative durations were recorded for both variables.

Definitions of the behaviour categories:

Passive behaviours: standing, sitting or lying down anywhere but at the door while alone (PASS-A), in the presence of the stranger (PASS-S), or in the presence of the owner (PASS-O).

Door-distress: displaying behavioural signs of distress while staying close to the door; active behaviours resulting in physical contact with the door (scratching, jumping at, etc.) and/or vocalising (i.e. barking, growling, howling, whining) in the close proximity (<1 m) of the door while alone (D/DISTR-A), in the presence of the stranger (D/DISTR-S), or in the presence of the owner (D/DISTR-O).

Door-passive: staying (standing, sitting, or lying down) in the close proximity of the door (<1 m) without physical contact with it, and/or vocalisation while alone (D/PASS-A), in the presence of the stranger (D/PASS-S), or in the presence of the owner (D/PASS-O).

Inter-observer agreement was assessed by parallel evaluation of the behaviour of 20% of the total sample by two independent coders who were blind to the conditions. The analysis of inter-observer agreement yielded a very good inter-observer reliability (Cohen's kappa values; PASS: 0.92, D/DISTR: 0.87, D/PASS: 0.91).

2.1.5. Data analysis

The relative percentage of the time spent in the above behaviours was calculated for the statistical analyses. Variables did not have Gaussian distribution (Kolmogorov–Smirnov test). At first we analysed the data with Generalised Estimating Equation (GEE) which is an extension of the GLM algorithm to accommodate the modelling of repeated measurement following non-normal distribution (Hardin and Hilbe, 2003). We employed a GEE analysis to examine the effect of the trial (1st, 2nd, 3rd conditioning and test trials) as within-subject factor and the effect of the group (*Conditioned* vs *Control*) as between subject-factor on the owners' opinion about the treatment. GEE analysis was also employed to examine the effect of the repetition (1st, 2nd and 3rd conditioning trials) as within-subject factor and the effect of the pre-treatment (administering Sedalin vs. vitamin) as between subject-factor on passive behaviour of dogs during the Conditioning trials. To analyse the effect of the conditioning we used GEE analysis to examine the effect of the trial (Baseline vs. Test) as within-subject factor and the effect of the pre-treatment (administering Sedalin vs. vitamin) during the Conditioning trials as between subject-factor on the dogs' behaviour. When GEE analysis revealed significant trial × treatment interaction, we calculated the change in the dogs' behaviours from Baseline to Test trials. We assumed that the difference in the relative durations of separation distress related behaviours expressed by the Sedalin conditioned dogs would be an indicator of subjects' susceptibility to the placebo effect. We subtracted the relative duration (time%) of a given behaviour in Baseline from the relative duration of that behaviour in Test trial. The 'difference values' of the *Conditioned* and *Control* groups were compared with Mann–Whitney *U* tests.

SPSS version 18 software was used for statistical analyses.

2.2. Experiment 2: Cognitive bias

2.2.1. Subjects

Twenty-one dogs (mean age ± SD: 3.3 ± 2.02 years, 11 males and 10 females, from 9 different breeds and 8 mongrels) from the 28 subjects that participated in Experiment 1 were called back for Experiment 2, 1–26 months after the first experiment. (One dog from the *Conditioned* group and

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Table 1
Experimental design of Experiment 1. All types of pre-treatments contained the additional water spraying and a piece of liverwurst right before the trials.

	Baseline trial	Conditioning (trials 2–4)	Test trial
Conditioned group ($N = 14$)	*Separation* No pre-treatment	*No separation* Sedative pre-treatment (Sedalin) Water spray	*Separation* Non-sedating pre-treatment (vitamin) Water spray
Control group ($N = 14$)		*No separation* Non-sedating pre-treatment (vitamin) Water spray	

six dogs from the *Control* group of Experiment 1 were not available any more.)

2.2.2. Procedure

The procedure was based on the study of Mendl et al. (2010). The Cognitive Bias Test was conducted in the same room as Experiment 1, the owner and an experimenter were present with the dog throughout the test. At the start of each trial, the owner led the dog to the starting position while the experimenter, standing behind the dog and the owner, baited (or did not bait, depending on trial type) a plastic pot (11 cm high, 14 cm in diameter) with a piece of sausage (see Fig. 2).

2.2.2.1. Training trials. Dogs were first trained that, when the pot was placed at one ('positive' – P) location, it contained food, and when it was placed at another ('negative' – N) location, it was empty. The locations were equidistant from the dog. For 11 dogs, P location was on the right hand side, and for 10 dogs it was on the left. The training always started with four warm up trials; two P trials (baited pot placed at the P location), when dogs could see the baiting, and two N trials (non-baited pot placed at the N location), in which the experimenter showed the empty container to the dog.

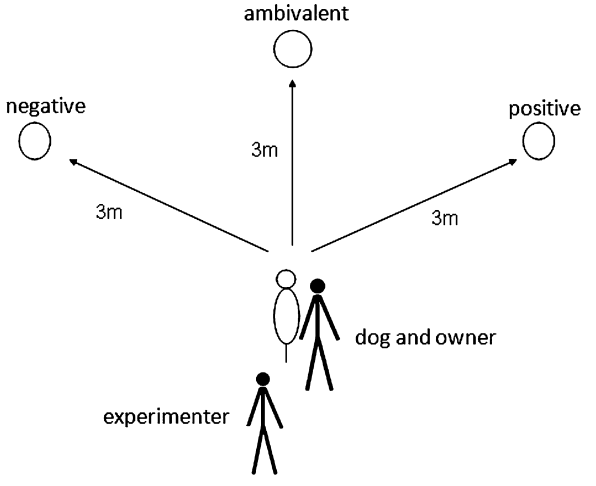

Fig. 2. Experiment 2: arrangement of the cognitive bias test. The experimenter standing behind the dog and the owner baited (or did not bait, depending on trial type) a plastic pot with a piece of sausage. Then she placed the food bowl at one of three pre-determined locations (negative, ambivalent, positive), then she went behind the owner, and the dog was released to approach the bowl.

Subsequently, P and N training trials were presented in a pseudorandom order, with no more than two trials of the same type being presented consecutively. Importantly however, in these trials, dogs were prevented from witnessing whether the container was baited or not, since the experimenter baited (or not) the pot behind the dog while the owner gently prevented the dog from looking back. When the experimenter had placed the pot and returned to her position behind the owner, the dog was released and allowed to choose. Owners were allowed to encourage their dog (saying "You can go!"). Training trials continued until the latency for each of the last five N trials was longer than any of the latencies for the last five P trials. After the dog had reached this learning criterion, the test trials began.

2.2.2.2. Test trials. Testing began once the learning threshold was achieved. Test trials were identical to training trials except that in three cases the empty pot was placed at the 'ambivalent' location (A) equally spaced between the P and N locations (see Fig. 2). The ambiguous trials were followed by one P and one N trial (9 trials in total; e.g.: APN, ANP, APN) in random order.

The purpose of the test trials was to investigate how dogs responded to the ambivalent location and whether they tended to approach them with a speed more similar to that at P location (indicating anticipation of a food reward – an 'optimistically' biased judgement of the ambivalent cue) or N location, that is, more slowly (indicating lower anticipation of food – a 'pessimistically' biased judgement).

2.2.3. Data analysis

Considering the wide range of time that elapsed between Experiments 1 and 2, we checked the data for any association with this duration (Pearson correlation test) to determine if the conditioning of the subjects might have had an effect on the expectancy scores.

The latency to reach the pot was defined as the time that elapsed between being released by the owner and the moment the dog put its head into the pot, or touched it with its nose. Latency was recorded for each trial. If the dog did not approach the container within 30 s, the trial was terminated, a latency of 30 s was allocated, and the next trial was initiated. Mean latencies followed normal distribution (Kolmogorov–Smirnov test).

Based on the study by Mendl et al. (2010), a *positive expectancy score* was calculated for each dog. That is, we adjusted each dog's mean ambivalent trial latencies ($M_{lat}A$) by taking into account its mean 'baseline' latencies to get to

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the positive ($M_{lat}P$) and negative ($M_{lat}N$) locations during the test phase as follows:

$$\text{positive expectancy score} = \frac{(M_{lat}N - M_{lat}A)}{(M_{lat}N - M_{lat}P)} \times 100$$

Higher scores indicate stronger positive expectancies. Positive expectancy scores followed normal distribution (Kolmogorov–Smirnov test).

Based on the results of Experiment 1, the individual placebo response could be best indicated by the relative change in the door-distress variable in Episode 1 (D/DISTR-A in the Baseline vs. Test trial). Higher relative changes are supposed to represent stronger placebo responses so the relative change of this value was calculated for each dog.

As the relationship between the placebo response values and positive expectancy scores was not linear, a logarithmic transformation was made on the placebo response values, thus the relationship could be analysed with Pearson-correlation.

3. Results

3.1. Experiment 1: conditioned placebo effect

3.1.1. Dogs' behaviour during the conditioning trials

As the owners were present in the experimental room throughout these trials, it is not surprising that only few dogs (4 in the 'Conditioned' and 3 in the 'Control' groups) displayed any behavioural signs of distress. Dogs spent hardly any time with distress behaviours; on average 0.5% (Sedalin group) and 0.65% (Control group) of the total duration, and, this remained extremely low even after repeated trials (0.2–1% of time during the 1st, 2nd and 3rd conditioning trials in both groups). However, dogs spent much more time with passive behaviours (on average 31% and 28% in the *Conditioned* and the *Control* groups respectively) and there was no effect of repetition (treatment: $\chi^2 = 0.2$, $p = 0.655$; repetition: $\chi^2 = 4.796$, $p = 0.091$).

3.1.2. Owners' evaluation of treatment effects

Although we did not find significant effects of Sedalin treatment on the recorded behaviour variables, the owners in the *Conditioned* group thought more often compared to the *Control* group that their dog received Sedalin gel in the conditioning trials (GEE analysis, group effect: $\chi^2 = 4.023$, $p = 0.045$; trial effect: $\chi^2 = 5.973$, $p = 0.113$; interaction: $\chi^2 = 2.816$, $p = 0.421$).

3.1.3. Dogs' behaviour in the Test vs. Baseline trials: the effects of conditioning

Separation episode (Episode 1)

During the separation episode dogs' passive behaviour was influenced by interaction between the trial and treatment (GEE, $\chi^2 = 6.537$, $p = 0.011$) with no significant main effects of the factors (trial: $\chi^2 = 0.356$, $p = 0.551$; treatment: $\chi^2 = 0.016$, $p = 0.901$). The change from Baseline to Test trials in the *Conditioned* group was positive and significantly different from the slight negative change in the *Control* group (Mann–Whitney test, $U_{(26)} = 50$, $p = 0.027$) (Fig. 3). Concerning passive behaviours close to the door, however, GEE analysis did not show significant main effects or interaction (trial: $\chi^2 = 0.239$, $p = 0.625$; treatment: $\chi^2 = 0.017$, $p = 0.896$; interaction: $\chi^2 = 1$, $p = 0.317$). The analysis of behavioural signs of distress close to the door showed a significant interaction between the trial and treatment (GEE, $\chi^2 = 4.66$, $p = 0.031$) with no main effects of trial (Baseline vs. Test: $\chi^2 = 0.001$, $p = 0.985$) or treatment (Sedaline vs. vitamin: $\chi^2 = 0.481$, $p = 0.488$) (Fig. 4). We found significant difference between changes

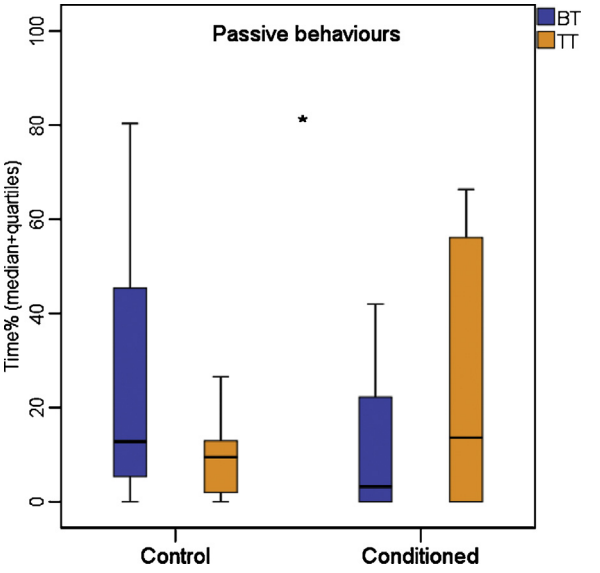

Fig. 3. Relative duration of passive behaviours in Episode 1. Dogs in the two groups showed different changes in passive behaviour after the conditioning. * indicates significant ($p < 0.05$) trial (Baseline vs. Test) × treatment (administering Sedalin vs. vitamin) interaction (BT = baseline trial, TT = test trial).

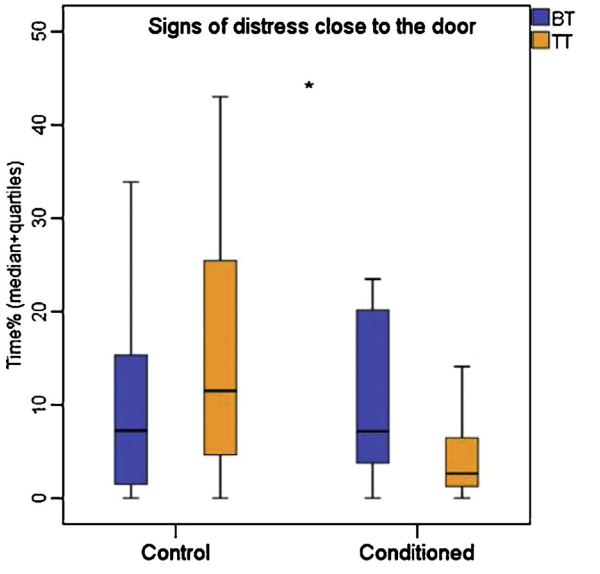

Fig. 4. Relative duration of distress close to the door in Episode 1. Dogs in the two groups showed different changes in distress signs close to the door after the conditioning. * indicates significant ($p < 0.05$) trial (Baseline vs. Test) × treatment (administering Sedalin vs. vitamin) interaction (BT = baseline trial, TT = test trial).

Table 2
Summary of the statistical analyses (separation episode, Experiment 1).

	GEE analysis (based on raw data)			Mann–Whiney test (change from baseline to test trial)
	Trial (Baseline vs. Test)	Treatment group (Conditioned vs. Control)	Trial × treatment interaction	Conditioned vs. Control group
D/distr-A	$\chi^2 = 0.001$, $p = 0.985$	$\chi^2 = 0.481$, $p = 0.488$	$\chi^2 = 4.66$, $p = 0.031$	$U_{(26)} = 48$, $p = 0.021$
PASS-A	$\chi^2 = 0.356$, $p = 0.551$	$\chi^2 = 0.016$, $p = 0.901$	$\chi^2 = 6.537$, $p = 0.011$	$U_{(26)} = 50$, $p = 0.027$
D/PASS-A	$\chi^2 = 0.239$, $p = 0.625$	$\chi^2 = 0.017$, $p = 0.896$	$\chi^2 = 1$, $p = 0.317$	$U_{(26)} = 79$, $p = 0.401$

in the *Conditioned* and the *Control* group (Mann–Whitney test, $U_{(26)} = 48$, $p = 0.021$; Fig. 5). Results of the separation episode are summarised in Table 2.

Episodes 2 and 3

There were no significant main effects or interactions for any of the behaviour variables in those episodes when the owner or the experimenter was present (GEE analyses, PASS-S: trial: $\chi^2 = 0.232$, $p = 0.627$; treatment: $\chi^2 = 0.052$, $p = 0.819$; interaction: $\chi^2 = 0.609$, $p = 0.435$; D/PASS-S: trial: $\chi^2 = 0.061$, $p = 0.804$; treatment: $\chi^2 = 0.551$, $p = 0.458$; interaction: $\chi^2 = 0.055$, $p = 0.815$; D/DISTR-S: trial: $\chi^2 = 0.069$, $p = 0.793$; treatment: $\chi^2 = 2.667$, $p = 0.102$; interaction: $\chi^2 = 1.736$, $p = 0.188$; PASS-O: trial: $\chi^2 = 2.291$, $p = 0.130$; treatment: $\chi^2 = 0.657$, $p = 0.418$; interaction: $\chi^2 = 1.863$, $p = 0.172$; D/PASS-O: trial: $\chi^2 = 0.716$, $p = 0.398$; treatment: $\chi^2 = 0.344$, $p = 0.558$; interaction: $\chi^2 = 2.270$, $p = 0.132$); D/DISTR-O: trial: $\chi^2 = 0.905$, $p = 0.342$; treatment: $\chi^2 = 0.816$, $p = 0.366$; interaction: $\chi^2 = 1.249$, $p = 0.264$).

These results show that the two types of treatment during the conditioning phase of the experiment affected dogs' later behaviour differently. After having received treatment with placebo (non-sedating vitamin) before the Test trial, the behaviour of dogs in the 'dog alone' episode depended on whether they had been treated with sedative substances during the conditioning phase.

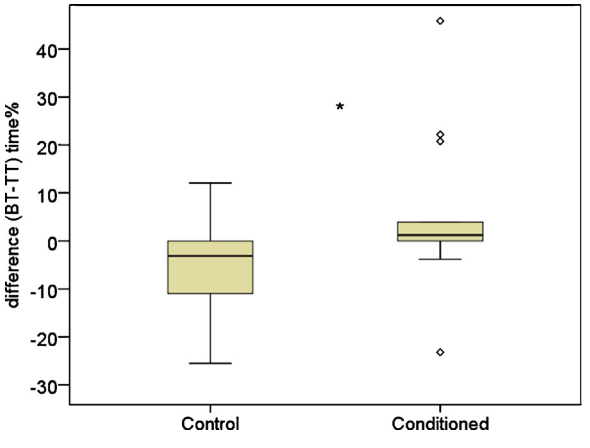

Fig. 5. Difference values of sign of distress close to the door. Dogs in the *Conditioned* group have higher difference scores (compared to the *Control* group), which represent higher placebo response (higher change in distress). * indicates significant ($p < 0.05$) between group difference (median + quartiles + outlier data).

3.2. Experiment 2: Cognitive bias

Subjects reached the training criterion on average after 30 trials (range 12–57 trials), and P and N locations were strongly differentiated also in the test trials; dogs approached the plastic pot sooner in P than in N type test trials (paired sample *t*-test, $t_{(20)} = 4.036$, $p < 0.001$). The positive expectancy scores ranged from −12.36 to 1179.5 (mean ± SD: 124.67 ± 243.79). There was no association between the time elapsed since the conditioning of the dogs in Experiment 1 and the expectancy scores (Pearson correlation test, $r_{(20)} = 0.335$, $p = 0.149$). We revealed a significant positive relationship between the positive expectancy scores and placebo response values in case of the *Conditioned* group (Pearson correlation test, $r_{(12)} = 0.697$, $p = 0.008$, Fig. 6) but not in the *Control* group ($r_{(7)} = 0.268$, $p = 0.521$).

These results indicate an association between 'cognitive bias' and 'susceptibility to placebo conditioning' measures in dogs, suggesting that dogs that have stronger positive expectancies (are more "optimistic") tend to be more responsive to the stress relieving effects of placebo treatment after conditioning with an active substance.

4. Discussion

Our results provide the first behavioural evidence in dogs for the development of a conditioned placebo effect, an effect that is well-known in humans (Benedetti et al., 2003; Goebel et al., 2002) and in laboratory animals (Isaac

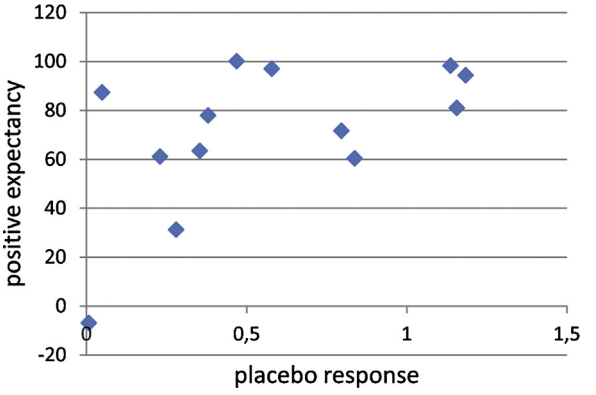

Fig. 6. Relationship between the individual placebo response and positive expectancy. There is a logarithmic relationship between the positive expectancy scores and the placebo response values ($r = 0.697$, $p = 0.008$) in the *Conditioned* group.

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and Isaac, 1976). In the two experimental groups (repeated treatment with sedative drug vs. non-sedating vitamin) we observed opposite trends of changes in separation anxiety related behaviours. The effects of sedative drug conditioning manifested itself via increased passivity and decreased duration of behavioural signs of distress displayed close to the door. In contrast, dogs in the control group showed an opposite tendency in these responses. Considering that using a double dose of Acepromazine (compared to our design), Tontodonati et al. (2007) could not find any physiological or behavioural effects 16 h after the treatment, long-term effects of acetylpromazin (Sedalin) are unlikely to explain the behaviour changes of the *Conditioned* group.

Importantly, owners were present throughout the conditioning trials in order to avoid any possibility of creating direct association between the separation from the owner and the anxiolytic effects of Sedalin. During the conditioning trials dogs had the opportunity to learn about the 'relaxed nature' of the environment but they had no opportunity to learn how to cope with separation distress under the influence of Sedalin. This procedure was designed to eliminate the possibility that dogs develop reduced behaviour signs of distress as a conditioned response. In the test trial only one aspect of the conditioning environment was changed: the presence/absence of the owner. In this new context the associative memory traces regarding the anxiolytic effects of Sedalin could have been mediated by the procedural aspects of the placebo administration and/or by the cues of the testing environment.

Our finding fits neatly into the placebo conditioning framework (McMillan, 1999); therefore we assume that the repeated experience with the effects of Sedalin, as an unconditioned stimulus, could have resulted in the formation of a relaxed inner state, which was associated with some characteristic property of the pre-treatment procedure and/or with some environmental cues of the experimental set up as conditioned stimuli. As a result of this associative process, treatment procedure with the same features but without administration of Sedalin could reduce some behavioural signs of separation distress. It is worth mentioning that we found no relevant differences between the *Conditioned* and *Control* groups in those episodes of Test trial in which the owner or the experimenter were present (Episodes 2 and 3). This suggests that the placebo effect, as a conditioned response, was specifically associated with the separation from the owner, despite the fact that separation anxiety was not triggered during conditioning trials where dogs were not separated.

These findings are in line with the notion that a wide range of placebo phenomena, even in humans, is often nothing more than "contextual healing" (Miller and Kaptchuk, 2008; Di Blasi and Kleijnen, 2003) because, in addition to the medicine or treatment, the situational context of the healing (environmental cues and the ritual of the treatment) can also play a crucial role in the process (Kaptchuk, 2002).

The significant conditioning effect in the Sedalin group was evident even though our placebo conditioning method had some practical limitations. The liverwurst might not be an ideal specific signal for the sedative drug, and the late sedative effect might also impair the formation of an association. We hoped to overcome these potential problems using the water spray procedure. In fact, spraying the dogs' muzzle and paws with water can be perceived as a salient and unusual stimulus event that could potentially be a key component of R-S learning during the conditioning phase, and thus a good mediator of the placebo effect. Using more stimuli, we cannot assess to what extent the different components of the treatment triggered the placebo effect, because any combination of them could be associated with the sedative state. The effect of the Sedalin gel could also vary among and even within subjects. Additionally, a relatively long time passed between the baseline and the test trials and there were relatively few, only three, conditioning trials (we should note that the number of trials affects the placebo-response in case of humans, see e.g. Colloca et al., 2010). Although owners had no preliminary information about which type of treatment their dogs received, we cannot exclude that they had some expectation regarding the treatment. However, since owners were not present during the separation episode, this could have an indirect (if any) effect on the dogs' behaviour.

Despite the above-mentioned potential confounding factors, our results provide strong support for the existence of a conditioned placebo effect in dogs because the assessment of the behavioural change was based on behaviour observations and not on the owners' report (c.f. Muñana et al., 2010; Jaeger et al., 2005; Cracknell and Mills, 2008). It is also worth mentioning that our findings concerning the conditioned placebo effect in alleviating separation anxiety have some veterinary implications and can be used to improve owners' and their dogs' daily life. Severe cases of separation anxiety often require the use of medications in addition to a behaviour modification programme. Once the desired effect is achieved, the dose of the medicine may be gradually reduced and finally merely the procedure can maintain the effect. However, so far the administration method of the medicine has not been considered as important. Our results suggest that applying a specific regimen, that is, administrating the medicine always with the same environmental cues, for example with the same specific food type and with a set ritual, the real medicine can later be effectively replaced by placebo. As the anxiety relieving effect of placebo conditioning in dogs is of great applied importance, more research is needed to get a better perspective on the most efficient aspects of the treatment and the situational context that contributes to the manifestation of the placebo effect.

The results of Experiment 2 expand our knowledge on placebo conditioning in dogs and highlight the potential importance of expectancy bias on the formation of placebo responses. The finding that dogs that were more responsive to the placebo treatment tended to show stronger positive expectancy in an ambivalent situation seems to be consistent with the conclusions of human studies (Geers et al., 2005, 2007, 2010; Morton et al., 2009). Importantly however, these human studies investigated the expectancy based and not the conditioned placebo effect. Although it remains unclear whether conscious learning (Stewart-Williams and Podd, 2004; Kirsch, 1985) or some 'cognitively blind' physiological response plays a more prominent role in the observed placebo effect, the

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association between dogs' positive expectancy scores and the magnitude of placebo-induced responses suggests that the observed placebo effect could not be entirely explained by unconscious factors.

In sum, the combined results of the two experiments open the door for studying the mechanism of placebo responses in the dog in its own right and provide further support for the validity of the application of the dog as a model species towards a better understanding of some aspects of the placebo phenomena in humans.

Conflict of interest

None.

Acknowledgments

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